IMMUNOHISTOCHEMICAL EVALUATION OF p-mTOR (PHOSPHORYLATED MAMMALIAN TARGET OF RAPAMYCIN) IN ORAL SQUAMOUS CELL CARCINOMA

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CERTIFICATE

This is to certify that **Dr. ABIRAMI.M** Post Graduate student (2015-2018) in the Department of Oral Pathology and Microbiology, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319, has done this dissertation titled **"IMMUNOHISTOCHEMICAL EVALUATION OF p-mTOR (PHOSPHORYLATED MAMMALIAN TARGET OF RAPAMYCIN) IN ORAL SQUAMOUS CELL CARCINOMA"** under our direct guidance and supervision in partial fulfilment of the regulations laid down by **THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY**, Chennai – 600032 for **MASTER OF DENTAL SURGERY - (BRANCH-VI)** ORAL PATHOLOGY AND MICROBIOLOGY degree examination.

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DECLARATION

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Co-Guide **Guide & Head of department** **Signature of candidate**

ABSTRACT

BACKGROUND

Oral cancer is one of the leading causes of cancer death in India. Due to persistence or recurrence of cancer despite aggressive therapy, there is a necessity for the need of treatment modalities that can target the molecules in the signaling pathway that are activated in cancer conditions. The Mammalian Target of Rapamycin (mTOR) is a member of the Phosphatidylinositol 3 (PI- 3)- Kinase-family & downstream molecule in the Akt /mTOR/pS6 signaling pathway which plays a critical role in regulating basic cellular functions including cell proliferation, cell survival, motility and angiogenesis. In carcinoma conditions, there will be dysregulation in the Akt /mTOR/pS6 signaling pathway. Hence, our present study is conducted to find the expression of p-mTOR in different grades of Oral Squamous Cell Carcinoma and to compare it with normal mucosa.

AIM:

The aim of this study is to analyse the Immunohistochemical expression of phosphorylated form of mTOR in different grades of OSCC in comparison with normal mucosa.

MATERIALS & METHODS:

This study was conducted on the archival retrieved formalin fixed, paraffin embedded tissues obtained from the Department of Oral Pathology, Adhiparasakthi Dental College and Hospital,

Melmaruvathur and from private hospitals. The study group included 21 cases of histologically diagnosed Oral Squamous Cell Carcinoma (7 cases of each Well, Moderately and Poorly differentiated squamous cell carcinoma). Control group includes biopsies from the normal buccal mucosa adjacent to the site of surgery during the surgical removal of third molar in 10 patients. 3 micron thickness sections were made from each sample and stained with p-mTOR primary antibody. The expression was scored and analysed using Kruskal Wallis test.

RESULTS:

Statistically significant difference ($p=0.03$) exists when comparing the intensity of staining of p-mTOR in between different grades of Oral Squamous Cell Carcinoma(OSCC). Increased intensity in the OSCC samples were observed when compared with normal mucosa. This value is also statistically significant ($p=0.003$). Also, when comparing the area of staining of p-mTOR in between different grades of OSCC statistically significant difference ($p=0.03$) exists across these groups. Increased number of positive stained cells were observed in OSCC samples when compared to normal mucosa and the result is statistically significant ($p=0.003$).

CONCLUSION:

We analysed the expression of p-mTOR as the first attempt in between different grades of OSCC and compared the expression with normal mucosa. Our present study reveals the significant expression of

p-mTOR in Akt /mTOR/pS6 signaling pathway which act as a prognostic marker or target for molecular therapies. Further research with a larger number of sample size with clinicopathologic correlation and long term follow up will emphasize more towards the use of mTOR as a prognostic marker. It will also be more interesting to correlate the presence and frequency of other molecular aberrations such as p-Akt and pS6 to trace out the role of entire Akt/mTOR/pS6 signaling pathway in OSCC. It will also be useful for the development of new therapeutic strategies targeting on mTOR pathway.

KEY WORDS: p-mTOR, Akt /mTOR/pS6 signaling pathway, Oral Squamous Cell Carcinoma, Immunohistochemistry.

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LIST OF ABBREVIATIONS

p-mTOR	-	Phosphorylated Mammalian Target of Rapamycin
OSCC	-	Oral Squamous Cell Carcinoma
IHC	-	Immunohistochemistry
ps6K	-	p70 ribosomal protein s6 kinase
PI3K	-	Phosphotidylinositol 3 kinase
Akt	-	Protein Kinase B
HNSCC	-	Head & Neck Squamous cell carcinoma
TSN	-	Tobacco specific nitrosamines
NNK	-	4-Nitrosomethylamino-1-(3-pyridyl)-1-butanone
XME	-	Xenobiotic Metabolising Enzymes
HPV	-	Human Papilloma Virus
DNA	-	Deoxyribonucleic Acid
HIV	-	Human Immunodeficiency virus
TSGs	-	Tumour suppressor genes
4E	-	Eukaryotic translational factor
mTORC1	-	mTOR complex 1
mTORC2	-	mTOR complex 2
GβL	-	Gamma Beta associated protein
LST	-	Lethal with SEC 13 protein
RAPTOR	-	Regulatory associated protein of mTOR
RICTOR	-	Rapamycin Insensitive component of mTOR
FK506	-	Tacrolimus
FKBP	-	FK506 Binding protein
PTEN	-	Phosphate and Tensin homolog detected on chromosome 10
RTK	-	Receptor tyrosine kinase
GPCR	-	G protein coupled receptor
4E-BP1	-	4E-Binding Protein1

TSC1	-	Tuberous sclerosis complex1
TSC2	-	Tuberous sclerosis complex 2
VEGF	-	Vascular Endothelial Growth Factor
HIF-1α	-	Hypoxia inducible factor 1 alpha
UCN-01	-	7-Hydroxystaurosporine
MT	-	Metallothionein
OLP	-	Oral lichen planus
OL	-	Oral leukoplakia
AML	-	Acute Myeloid Leukemia
ER	-	Estrogen recptor
PR	-	Progesterone receptor
TCL1B	-	T cell Leukemia/ lymphoma 1B
MAPK	-	Mitogen activated protein kinase
RAS	-	a small GTPase
ERK	-	Extracellular signal related kinase
MTC	-	Medullary Thyroid carcinoma
TGFBR1	-	Transforming growth factor binding receptor 1
PLB	-	Plumbagin
GSK3β	-	Glycogen synthase kinase 3 beta
ROS	-	Reactive oxygen species
HRP	-	Horse raddish peroxidase
DAB	-	Diamino benzidine
PBS	-	Phosphorylated buffered saline
TBS	-	Tris buffered saline
H_20_2	-	Hydrogen peroxide
DPX	-	Distyrene Plasticizer Xylene

INTRODUCTION

Squamous Cell Carcinoma is an epithelial malignancy that exists in organs that are normally lined by squamous epithelium including skin, lips, mouth, esophagus, urinary tract, prostate, lungs, vagina and cervix[1]. Oral Squamous Cell Carcinoma (OSCC) contributes to 95% of all forms of head and neck cancer and its incidence is increased by 50% in the past decade[2]. Dysregulation in the cell cycle and the proliferation of malignant cells leads to the loss of control mechanisms that ensure the normal function of tissues[3]. Advances in diagnosis and therapeutic techniques for these lesions have extracted novel molecular targets, uncovered signal pathway dominance and advanced early cancer detection[4].

In recent years, advances in molecular biology, revealed several steps in carcinogenesis related to signaling pathways and enables us to look in to the molecular events and aberrations which leads to the cancer development and progression. Akt/ mTOR /pS6 signaling pathway has been recognised as one of the most commonly associated pathways in different types of human cancer, including OSCC[5].

The diagnosis of cancer relies on the analysis of tissue and cytology specimens which is obtained through various procedures. When a cell become cancerous, new antigens which are unfamiliar to the immune system appears on the cell surface. The immune system

recognises these new antigens called tumour antigens as foreign and may be able to contain or destroy the cancerous cells[6].

Tumour marker is a molecule that indicates the likely presence of cancer or can also be defined as one which provides information about the likely future behaviour of an existing cancer (Eg – ability to metastasize or to respond to therapy). Most of the existing tumour markers are chiefly useful in making a clinical decision after initial suspicion of cancer or its behaviour which has been already raised by more conventional means[6].

mTOR (Mammalian Target of Rapamycin) is one of the major targets of activated Akt which in turn controls a number of downstream molecules, such as ribosomal protein pS6 that eventually regulates fundamental processes such as cell survival, proliferation, protein synthesis and angiogenesis[5]. In response to various stimuli such as nutrient, oxygen, insulin, growth factors, adenosine triphosphate and tobacco metabolites mTOR is activated by Phosphorylation of Ser 2448 via the Phosphatidylinositol 3-kinase (PI3-K)/ Akt signaling pathway. This leads to subsequent activation of key modulators which are involved in protein synthesis[7]. Dysregulations in upstream and downstream molecules of mTOR signaling takes place in 90-100% of HNSCCs (Head and Neck Squamous Cell Carcinomas) suggesting that markers and targets in the Akt/mTOR/pS6signaling pathway are especially of clinical significance[5]. p-mTOR is an important

therapeutic target for Oral Squamous Cell Carcinoma and an independent prognostic factor in the overall survival (OS) to assess high risk subgroups and as a guide to therapy[7].

The purpose of this study is to analyse the Immunohistochemical expression of phosphorylated form of mTOR in different grades of OSCC in comparison with normal mucosa, in order to assess the potential contribution of Akt/mTOR/pS6 signaling pathway aberrations in OSCC.

AIM AND OBJECTIVES

AIM:

To analyse the Immunohistochemical expression of phosphorylated form of mTOR (p-mTOR) in different grades of OSCC in comparison with normal mucosa.

OBJECTIVES:

1. To evaluate the immunohistochemical expression of p-mTOR in different grades of Oral Squamous Cell Carcinoma
2. To compare the immunohistochemical expression of p-mTOR in different grades of Oral Squamous Cell Carcinoma
3. To compare the immunohistochemical expression of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa
4. To evaluate the area of staining of p-mTOR in different grades Oral Squamous Cell Carcinoma
5. To compare the area of staining of p-mTOR in different grades Oral Squamous Cell Carcinoma
6. To compare the area of staining of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa

REVIEW OF LITERATURE

ORAL SQUAMOUS CELL CARCINOMA (OSCC)

Squamous Cell Carcinoma is one of the most common malignant tumours of oral cavity. It constitutes about 90-95% of all oral malignancies[8].

ETIOLOGY:

The etiology of OSCC is multifactorial inclusive of both intrinsic & extrinsic factors. It is predicted that more than a single factor is necessary to produce malignancy. The etiological factors include tobacco smoking, smokeless tobacco, betel quid (paan), alcohol, phenolic agents, radiation, iron deficiency, vitamin A deficiency, syphilis, candidal infection, oncogenic viruses, immunosuppression, oncogenes and tumour suppressor genes[2,9,10].

Tobacco: Tobacco consumption continues to remain as the most important risk factor as it alone accounts for the millions of cancer death annually. The most important carcinogens in tobacco smoke are the aromatic hydrocarbon benz-pyrene and the tobacco – specific nitrosamines (TSNs) namely 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). Certain other classes of enzymes that are involved in the activation or degradation of carcinogens and procarcinogens are termed as Xenobiotic Metabolising Enzymes (XME). Hence, certain XME genotype may increase

individual susceptibility to cancer through erogenous carcinogen metabolism which leads to increased carcinogen exposure[2].

Betel quid**:** Betel quid (also referred to as pan or paan) contains betel leaf, areca nut, slaked lime and tobacco. Other ingredients are often added namely spices such as cardamom, cloves or aniseed to the quid in India. It is evident from the research studies that betel quid ingredients especially of tobacco and areca nut are having carcinogenic, mutagenic and genotoxic potential[2].

Alcohol: Consumption of alcohol has been shown to act synergistically with tobacco and having increased risk of development of oral cancer.[2]Alcohol may also act as a solvent and increase the penetration of carcinogens in to target tissues[4].

Viruses: Human Papilloma Virus (HPV) positivity is higher in tumours of oral cavity (59%), pharynx (43%) and larynx (33%).HPV subtypes 16 & 18 are more associated with malignant transformation[4,11]. Epstein-Barr viruses and HPV are associated with carcinogenesis in oropharyngeal cancers & nasopharyngeal cancers respectively[12].

Diet: Fruits and vegetables (high in vitamin A &C) are considered as protective in oral neoplasia, whereas meat and chilli powder are thought to be risk factors[4,13].

Family History of Head and Neck Squamous Cell Carcinoma (HNSCC): Family History of Head and Neck Squamous Cell Carcinoma is a risk factor. The ability to repair DNA which is damaged by tobacco carcinogens is defective in patients with family history of HNSCC[4].

Immune deficiency: A defective immune response, as seen in a Human Immunodeficiency Virus (HIV) infected individual, may predispose to cancer. OSCC of the lip is more common in transplant recipients receiving immunosuppressive therapy, but HIV infection does not predispose to intra-oral squamous cell carcinoma[4].

Molecular changes in Oral cancer: Cancer occurs through multiple steps, each step is characterised by the sequential accumulation of additional genetic defects, followed by clonal expansion. The genetic alterations observed in head and neck cancer are significantly due to oncogene activation and tumour suppressor gene inactivation which leads to de-regulation of cell proliferation and death[4].

Tumour Suppressor Genes(TSGs) and Growth Regulators: Growth regulators and TSGs act as transducers of negative growth signals. Genetic alterations involving the tumour suppressor genes p16 & p53, are frequently noted in head and neck tumours. The gene can be inactivated by several mechanisms such as point mutations, deletions and binding with cellular and viral proteins. p53 gene inactivation has been demonstrated in Squamous Cell Carcinoma[4].

CLINICAL FEATURES:

OSCC may take several clinical forms. It may look like a leukoplakia, a verrucous leukoplakia, an erythroleukoplakia or an erythroplakia, any of which may develop in to a necrotic ulcer with irregular, raised indurated borders or in to a broad based exophytic mass with a surface texture which may be verrucous, pebbled or relatively smooth. When traumatized, OSCC bleeds easily and most often becomes superficially secondarily infected. Large lesions may interfere with normal speech, mastication or swallowing[9]. OSCC can be similar in most studies, but can vary from country to country[14].

About two-thirds of OSCC are of substantial size and will have clinically detectable metastases to cervical lymph nodes at the time of diagnosis. The lymph nodes which are affected are firm and non - tender to palpation & fixed and matted lymph node has occurred if extracapsular spread invade in to the surrounding connective tissue. A high rate of local and regional recurrence, distant metastasis and mortality is associated with high rate of local recurrence[9].

HISTOPATHOLOGICAL FEATURES:

- ♠ SCC arises from dysplastic surface epithelium histopathologically characterised by the presence of invasive islands and cords of malignant squamous epithelial cells
- ♠ Irregular extension of lesional epithelium through the basement membrane and in to subepithelial connective tissue represents the invasion.

- ♠ Individual squamous cells & sheets or islands of cells are seen within the connective tissue without the presence of attachment to the surface epithelium.
- ♠ The underlying adipose tissue, muscle or bone may be invaded by dysplastic epithelial cells.
- ♠ Angiogenesis is induced by lesional epithelium and desmoplastic changes occurs occasionally.
- ♠ Lesional cells generally exhibits hyperchromatism & increased nuclear cytoplasmic ratio.
- ♠ Cellular & nuclear pleomorphism are seen at varying degrees
- ♠ Keratin pearls are evident & individual cell keratinisation may also occur [15].

GRADING:

Lesions are graded based on a 3-point (Grades I to III) or a 4-point (Grades I to IV) scale. The higher numerals denote the less differentiated tumours.

- ♠ **Well differentiated squamous cell carcinoma**: A tumour which closely resembles its parent tissue of origin, grow at a slower pace and metastasize later in its course is called low grade, grade I or Well differentiated squamous cell carcinoma.
- ♠ **Poorly differentiated squamous cell carcinoma:** A tumour which shows much cellular & nuclear pleomorphism, little or no keratin production & is difficult to identify its tissue of origin. It also metastasizes early in its course and termed as

high grade, grade III or IV, Poorly differentiated or Anaplastic squamous cell carcinoma.

- **Moderately differentiated squamous cell carcinoma:** A tumour which shows the microscopic appearance in between these two extremes are considered as grade II or Moderately differentiated squamous cell carcinoma[15].

Grading of squamous cell carcinoma – Broder's classification (1920):

- Grade I: Well differentiated tumours- 75-100% of cells are differentiated
- Grade II: Moderately differentiated tumours- 50-75% of cells are differentiated
- Grade III: Poorly differentiated tumours- 25-50% of cells are differentiated
- Grade IV: Anaplastic tumours- 0-25% of cells are differentiated[16].

mTOR (MAMMALIAN TARGET OF RAPAMYCIN)

The Mammalian Target of Rapamycin (mTOR) is a **serine - threonine protein kinase** and it is a **main downstream target of the PI3/Akt** (Phopsphotidylinositol 3 kinase /protein kinase B) **signaling pathway**[17,18]. It belongs to the PI3k related kinase family[7].This pathway plays its significant role in maintenance of overall cell survival, cell growth and cell proliferation[7]. In addition, mTOR is also involved in protein and lipid synthesis, mitochondrial metabolism, biogenesis and it negatively regulates autophagy[19]. In response to various stimuli such as nutrient, oxygen, insulin, growth factors, adenosine triphosphate & tobacco metabolites, mTOR gets activated by phosphorylation of Ser 2448 (Serine) via the PI3/Akt signaling pathway. The activated (phosphorylated) mTOR is known as p-mTOR. Ultimately this leads to the subsequent activation of three key modulators in protein synthesis and they are[7]:

- The eukaryotic translational factor,4E
- The p70 ribosomal S6 kinase and
- Elongation factor 2

mTOR complexes:

Two well defined multiprotein complexes have been identified and cloned: They are[20]:

1.mTOR complex 1 (mTORC1)

2.mTOR complex 2 (mTORC2)

mTORC1:

mTORC1 comprises of mTOR, GβL/LST8 and Regulatory Associated Protein of mTOR(RAPTOR). This complex promotes translation in response to different stimuli & maintains the timing of cell growth[12].

mTORC2:

mTORC2 comprises of mTOR, GβL and Rapamycin Insensitive Component of mTOR (RICTOR). This complex stimulates cells to increase in their size and mass[12].

Both of the mTOR complexes function significantly in cytoplasm. Higher eukaryotes (mammals, flies & worms) contain only one TOR gene[12].

RAPAMYCIN & RAPALOGS

Rapamycin is an antibiotic obtained from the bacterium *Streptomyces hygroscopicus* which is found in soil of Easter island[21]. Rapamycin was discoverd as a potent antifungal agent, but it is also having the immunosuppressive effect. The immunosuppressant FK 506 (Tacrolimus) and rapamycin are having similar chemical structures and both can binding to the same intracellular receptor, FKBP12. Eventhough tacrolimus and rapamycin are binding to the same protein, they act by different mechanism of actions in cells. In addition to rapamycin, three rapamycin analogs (rapalogs) are identified now in humans. Rapalogs are having the same mechanism of action as rapamycin. Rapamycin has also been proposed to inhibit mTOR by destabilising the mTOR –Raptor complex. Inhibition of mTOR kinase activity is more effective for killing cancer cells which exhibits increased mTOR signaling[22].

mTOR KINASE INHIBITORS

A small molecule which is formulated to compete with ATP in the catalytic site of mTOR inhibits all of the kinase dependent functions of mTORC1& mTORC2. mTOR kinase inhibitors are formulated only to inhibit class I PI3 Kinases. So, the drugs which are inhibiting both the PI3K/mTOR are having the therapeutic advantage[22].

Inhibition of mTOR dependent signaling would reduce the cell growth & cell proliferation. The drug named rapamycin (clinically

known as sirolimus) specifically inhibits TOR, which results in reduced cell growth, cell cycle progression and reduce the rate of cell proliferation. Thus, rapamycin and its analogues (rapalogs) are currently being tested in clinical trials to treat various carcinomas[2].

mTOR DEREGULATION IN ORAL SQUAMOUS CELL CARCINOMAS

Overexpression of mTOR protein occurs as a result of combination of upstream oncogene activation and downregulation of suppressor gene[14]. PTEN (Phosphate and Tensin homolog detected on chromosome TEN) is a tumour suppressor gene. The major substrate for PTEN is PIP3 (Phosphotidylinositol 3,4,5 triphosphate), a product of PI3/Akt signaling pathway. Therefore, the loss of PTEN function leads to increased levels of PI3 and hyperactivation of mTOR in oral squamous cell carcinomas. The dysregulated mTOR expression was also reported on esophageal, salivary gland, hepatic, gastric, breast, bladder, biliary tract and lung cancers[7].

PI3-Akt-mTOR SIGNALING PATHWAY

The PI3 pathway is the main signal transduction system which links the oncogenes and multiple receptor classes for many essential cellular functions. It is considered as the most commonly activated signaling pathway in human cancer. This pathway acts both as an opportunity and a challenge for cancer therapy. Receptor tyrosine kinases (RTKs), G protein coupled receptors (GPCRs), PI3Ks act as major downstream effectors and transduce signals from different Growth factors and cytokines in to intracellular messages by generating phospholipids, which in turn activates the serine/Threonine kinase Akt and other downstream effectors in the pathways. The tumour suppressor gene PTEN negatively regulates the PI3K pathway. Recent human cancer genomic studies have showed that many components of PI3K pathway are often targeted by germline or somatic mutations in a broad spectrum of human cancers. Based on these findings and the fact that PI3K and other kinases in the PI3K pathway are effectively suited for pharmacologic intervention, this pathway is considered as one of the most attractive targets for therapeutic intervention in cancer[24].

PI3Ks have been divided into three classes based on their structural characteristics and substrate specificity. Of these, the most commonly studied are class I enzymes which are activated directly by cell surface receptors. Class I PI3Ks are further subdivided into **class IA enzymes**, activated by RTKs, GPCRs and certain oncogenes like small G protein Ras and **class IB enzymes,** which is regulated specifically by GPCRs[24].

The PI3K-Akt - mTOR signaling in cancer cell growth and survival

The family of lipid kinases termed PI3K are considered as key regulators in various essential cellular processes such as cell survival, growth and differentiation. The PI3K pathway has various important nodes that play a significant role in this pathway which results in a diversity of functional outcomes. The Akt -mediated activation of downstream targets, including mammalian target of rapamycin (mTOR) increases cell proliferation and the regulation of translation in response to growth factors by the phosphorylation of the protein synthesis machinery. This translational promotion by mTOR includes the **phosphorylation of ribosomal protein S6 kinases (s6K) and 4E-binding protein 1 (4E-BP1)** the latter of which ultimately results in release of eukaryotic translation initiation factor 4E (eIF4E), which has known anti-apoptotic activities. These effects are counteracted by tuberous sclerosis complex-1 (TSC1) -TSC2 complex, which has inhibitory effects on 4E-BP1 and eIF4E. Akt also phosphorylates and inhibits TSC2, which depicts the complexity of this pathway. Rapamycin and its analogues are capable of inhibiting mTOR, but this can result in activation of upstream proteins such as Akt, due to the loss of a feedback loop mechanism. In addition, the PI3K-Akt pathway also interacts with complex molecular mechanism which controls cellular energy control and glucose metabolism. The PI3K signaling also controls growth, proliferation, senescence and angiogenesis. These processes are mediated by vascular endothelial growth factor

(VEGF) transcriptional activation and hypoxia- inducible factor-1alpha (HIF-1α) expression[25].

The PI3-KINASE /AKT signaling pathway delivers an anti-apoptotic signal:

Inhibition of PI 3-kinase accelerates apoptosis and an activated form of the serine/threonine kinase Akt, a downstream effector of PI3-kinase blocks apoptosis[26].

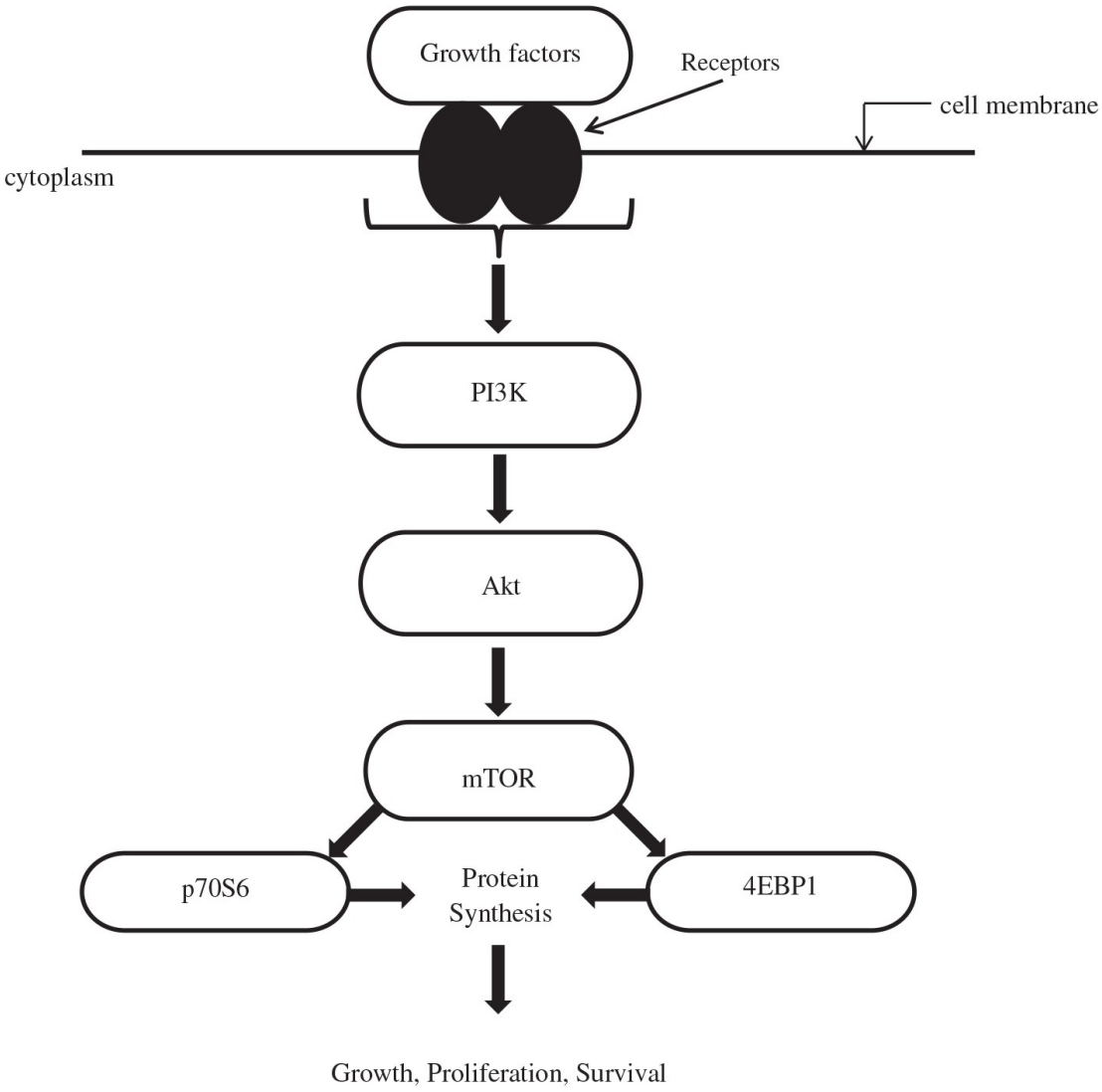


Figure 1. mTOR pathway

Figure depicts that the Growth factors & their receptors send signals to PI3K (Phosphotidylinositol 3-kinase) which send downstream signals to Akt (Protein kinase B) which send transmit signals to mTOR (Mammalian Target of Rapamycin). mTOR phosphorylates & activates p70S6K (ribosomal kinase) and inhibits 4 EBP1 (translational factor binding protein). The overall pathway plays important role in maintaining protein synthesis, cell growth and cell survival[27,28,29].

EXPRESSION OF p-mTOR IN HNSCCs & IN ORAL LESIONS

Panomwat Amornphimoltham *et al* in 2004 evaluated the status of activation of Akt in various stages of Squamous Cell Carcinoma development in mice and in clinical samples from HNSCC patients. By immunohistochemical analysis, using a recently developed phosphorylation state-specific antibody, they depicted that Akt activation correlates closely with the progression of mouse skin squamous cell carcinoma. They also observed that activation of Akt is a frequent event in human HNSCC because active Akt can be detected in these tumors with a pattern of expression and localization which correlates with the progression of the lesions. Based on these observations, Akt was constitutively activated in a large fraction of HNSCC-derived cell lines. They also provide evidence that the Akt signaling pathway may represent a biologically relevant target for a novel antineoplastic agent, UCN-01 (7-hydroxystaurosporine), which recently is shown to be active in cellular and xenograft models[30].

H. A. R. Pontes *et al* in 2009 did a study to evaluate the immunoexpression of p-Akt and Metallothionein (MT) proteins in dysplasic and neoplasic oral lesions. Immunohistochemical studies were carried out on 10 normal epithelium, 30 OL and 15 OSCC paraffin-embedded samples. Immunoperoxidase reactions for p-Akt and MT proteins were applied on the specimens and the positivity of the reactions were calculated for 1000 epithelial cells. A significant difference was observed in the immunoexpression for p-Akt and MT

when the OSCC samples were compared with normal and dysplastic epithelial groups. Based on the data obtained, p-Akt and MT activation may play an important role in the conversion of a potentially malignant oral lesion to a malignant lesion[31].

Ho-Tai Wu *et al* in 2009 evaluated the role of phosphorylated Akt (p-Akt) in oral carcinogenesis induced by nicotine and alkaline environments. By using Immunohistochemistry, (IHC) p-Akt expression in cancerous (n = 30) precancerous (n = 30), and normal mucosa tissues (n = 10) were studied. Higher p-Akt expression was noted in cancerous group than in normal mucosa (P = 0.0002) and precancerous (P = 0.0049) groups. The dose-dependent increase in p-Akt by nicotine treatment was specifically observed. Higher p-Akt expression was also expressed in more alkaline environment[32].

Cheryl Clark *et al* in 2010 evaluated the biomarkers mTOR, Akt, 4EBP1, and S6 kinase, signaling components in HNSCC patients and compared it with normal mucosa. Expression of phosphorylated Akt and phosphorylated mTOR were significantly increased in tumors compared to non-cancer oral mucosa samples (p=0.004 and p=0.026 respectively). p-mTOR and p4EBP1 expression were higher in patient junctional zones (p=0.017 and p=0.022 respectively) and no difference in p-AKT or p-S6 expression in HNSCC patients junctional zone compared to tumors. IHC revealed p-mTOR expression was 81.9% sensitive and 100% specific in differentiating cancer from non-cancer mucosa, while p-4EBP1 expression by IHC was only 50.0% sensitive

and 95.5% specific for differentiating normal mucosa from HNSCC ($p<0.01$)[33].

Georgios Prodromidis *et al* in 2013 evaluated the activation status of Akt, mTOR, and pS6 in oral lichen planus (OLP) and compared it with oral premalignant and malignant lesions and normal oral mucosa (NM). Immunohistochemistry for p-Akt, p-mTOR, and phospho-pS6 was performed in 40 OLP, 20 Oral Leukoplakias (OL), 10 OSCC and 10 control samples of normal mucosa. Nuclear p-Akt expression was observed in majority of cases in all categories, being predominantly higher in OL. Cytoplasmic p-Akt and p-mTOR staining was observed only in few OLP cases, being significantly lower compared to OL and OSCC. Phospho-pS6 showed cytoplasmic positivity in majority of OLP cases, which however was significantly lower compared to OL and OSCC. They concluded that cytoplasmic p-Akt, p-mTOR and phospho-pS6 levels were lower in OLP when compared to OL and OSCC[5].

Luis Silva Monteiro *et al* in 2013 studied the expression of phosphorylated mammalian target of rapamycin (p-mTOR) and phosphatase and tensin homolog deleted on chromosome TEN (PTEN) in oral squamous cell carcinomas (OSCCs) and correlated them with clinicopathologic characteristics and prognosis. They studied p-mTOR and PTEN protein expression by immunohistochemistry in 72 cases of OSCCs. p-mTOR expression was noted in 46 (63.9%) cancers and PTEN expression was absent in 22 (30.6%). The p-mTOR was suggested as a reliable biological marker to identify high-risk

subgroups and they act as a guide to therapy. In addition, the higher expression levels of p-mTOR suggests that this protein may act as a promising therapeutic target for OSCC[7].

p-mTOR IN OTHER LESIONS

J Boone *et al* in 2008 investigated the expression of p-mTOR (Ser2448) immunohistochemically in formalin-fixed, paraffin-embedded Oesophageal SCCs (OSCCs). Normal oesophageal epithelium shows negativity for p-mTOR. Activated mTOR expression was located in the cytoplasm of oesophageal tumour cells. 26 (25%) of 105 assessable OSCCs expressed tumour cells with positive staining for activated mTOR. Activated mTOR expression was associated with a lesser degree of differentiation only ($p = 0.024$). No correlation was observed between p-mTOR and the proliferation marker Ki-67. He concluded that activated mTOR can be detected in one-quarter of OSCCs[34].

Alberto M Martelli *et al* in 2009 explained in his review about how PI3K/Akt/mTOR signaling network is constitutively active in acute myelogenous leukemia (AML). He concluded that the limit of acceptable toxicity for standard chemotherapy has been reached in AML and new therapeutic strategies are needed. Targeting the PI3K/Akt/mTOR signaling network with small molecule inhibitors, alone or in combinations with other drugs, may provide less toxic and more efficacious treatment of AML patients[35].

Sunil Badve *et al* in 2010 analysed the nuclear and cytoplasmic phospho-Akt (p-Akt) expression by immunohistochemistry in a breast cancer tissue microarray ($n = 377$) and corelated these data with the expression of estrogen receptor (ER) progesterone receptor (PR) and

FOXA1. Nuclear localization of p Akt (nuclear-p AKT) was associated with long-term survival (P 0.004). Within the ER, PR subgroup, patients with nuclear-p Akt positivity had better survival when compared to nuclear-p Akt–negative patients ($P < 0.05$). TCL1B (T Cell Leukemia/ Lymphoma 1 B) family proteins play significant role in regulating nuclear transport and/or activation of Akt. TCL1B is overexpressed in ER - positive compared with ER-negative breast cancers and in lung metastasis–free breast cancers[36].

Di Wang *et al* in 2011 analysed the significance of mammalian target of rapamycin (mTOR) and its active form, p-mTOR in colorectal carcinomas. Immunohistochemistry was used to find the expression of mTOR and p-mTOR proteins in 108, 40 and 40 tissue samples from colorectal carcinoma, normal colonic mucosa and adenomatous polyps samples, respectively. The correlation of mTOR and p-mTOR expression with clinicopathological characteristics of colorectal carcinoma was observed. The positive rates of mTOR and p-mTOR were significantly increased in colorectal carcinoma (61.1% and 61.1%, respectively, $p<0.05$) than in normal colonic mucosa (7.5% and 2.5%) and in adenomatous polyps (27.5% and 20%). Overexpression of total mTOR protein was associated with T1/T2 stage tumors, lymph node metastasis, distant metastasis) and degree of differentiation. In addition, p-mTOR overexpression was linked with degree of differentiation and TNM stage. The overexpression of mTOR and p-

mTOR may play important roles in colorectal carcinogenesis in relation to the degree of differentiation, invasiveness and metastasis[37].

Anna Tamburrino *et al* in 2012 analysed the phosphorylation status of proteins of the RAS/MEK/ERK and PI3K/Akt/mTOR pathways in 53 Medullary thyroid carcinoma (MTC) tissues (18 hereditary, 35 sporadic), including 51 primary MTCs and 2 cases with only lymph node metastases (LNM). In addition, they also analysed the functional relevance of the mTOR pathway by measuring cell viability, motility and tumorigenicity upon mTOR chemical blockade. Phosphorylation of ribosomal protein S6 (pS6), a downstream target of mTOR, was observed in 49 (96%) of 51 primary MTC samples. This was associated with activation of Akt (phospho-Ser473, S > 1) in 79% of cases studied. The Akt /mTOR pathway is activated in MTC, particularly, in LNMs[38].

Zhi-Jun Sun *et al* in 2012 evaluated the efficacy of rapamycin treatment in chemoprevention and chemotherapy of tumorigenesis in a genetically defined mouse model of head and neck squamous cell carcinoma (HNSCC). Knockdown of Tgfbr1 (Transforming Growth factor binding receptor 1) and /or PTEN in human HNSCC cell lines leads to activation of mTOR activity complex 1 and increased levels of survivin. Chemopreventive rapamycin treatment delayed the onset of the HNSCC tumors and prolonged survival in 2cKO mice significantly. These findings represent that, tumorigenesis in 2cKO HNSCC is associated with activation of the Akt/mTOR/survivin pathway and

inhibition of this pathway can be achieved by rapamycin treatment successfully[39].

Tjinta Brinkhuizen *et al* in 2014 did immunohistochemical staining of formalin-fixed, paraffin-embedded Basal cell carcinoma (BCC) (n = 45) and Trichoepithelioma (TE) (n = 35) samples to assess activity of HIF1 (Hypoxia inducible factor-1 alpha), mTORC1 and their most important target genes. They concluded that HIF and mTORC1 signalling seems to be active in both BCC and TE & there are no appreciable differences between the two with respect to pathway activity. HIF, mTORC1 and their target genes are not a reliable diagnostic tool for the discrimination of BCC and TE[40].

Shu-Ting Pan' *et al* in 2015 investigated the effects of plumbagin (PLB) on cell cycle distribution, apoptosis, and autophagy, and the underlying mechanisms in the human TSCC cell line SCC25. The results of their study revealed that PLB exerted potent inducing effects on cell cycle arrest, apoptosis, and autophagy in SCC25 cells. PLB inhibited phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), glycogen synthase kinase 3β (GSK3β), and p38 mitogen-activated protein kinase (p38 MAPK) pathways as represented by the alteration in the ratio of phosphorylation level over total protein expression level, which contributes to the inducing effect of autophagy. These results predict that PLB promotes cellular apoptosis and autophagy in TSCC cells

involving p38 MAPK and PI3K/Akt/mTOR-mediated pathways with contribution from the GSK3β and ROS-mediated pathways[41].

V Vasko *et al* in 2017 analysed 46 thyroid cancer, 20 thyroid follicular adenoma and adjacent normal tissue samples by immunohistochemistry for activated Akt (pAkt), Akt 1, 2 and 3 and p27 expression. Akt activation was noted in 10/10 follicular cancers, 26/26 papillary cancers and 2/10 follicular variant of papillary cancers, but in only 4/66 normal tissue samples and 2/10 typical benign follicular adenomas. Immunoactive pAkt was highest in regions of capsular invasion and was localised to the nucleus in follicular cancers and the cytoplasm in papillary cancers, except for invasive regions of papillary cancers where it was localised to both regions. Immunoactive Akt 1, but not Akt 2 or Akt 3, correlated with pAkt localisation and nuclear pAkt, was associated with cytoplasmic expression of p27. In vitro studies using human thyroid cancer cells explained that nuclear translocation of Akt 1 and pAkt were associated with cytoplasmic p27 and cell invasion and migration. Cell migration and the localisation of Akt 1, pAkt and p27 were inhibited by PI3 kinase, but not by MEK (MAPK/ERK kinase) inhibition. They concluded that nuclear activation of Akt 1 plays significant role in thyroid cancer progression[42].

MATERIALS AND METHODS

STUDY DESIGN AND PATIENT SELECTION:

This study was conducted on the archival retrieved formalin fixed, paraffin embedded tissues obtained from the Department of Oral Pathology, Adhiparasakthi Dental College and Hospital, Melmaruvathur and from private hospitals. The study group included 21 cases of histologically diagnosed Oral Squamous Cell Carcinoma (7 cases of well differentiated squamous cell carcinoma, 7 cases of Moderately differentiated Squamous Cell Carcinoma and 7 cases of Poorly differentiated Squamous Cell Carcinoma). Control group includes biopsies from the normal buccal mucosa adjacent to the site of surgery during the surgical removal of third molar in 10 patients.

Two subsequent sections, each 3µ thickness were cut for each sample, from formalin fixed, paraffin embedded tissues of histologically diagnosed Oral Squamous Cell Carcinoma and normal buccal mucosa.

MATERIALS

PARAFFIN BLOCKS:

Paraffin embedded tissues of histologically confirmed cases of Oral Squamous Cell Carcinoma were used in the study.

EQUIPMENTS:

- Microtome (Thermo scientific, MICROM HM340E)
- Paint brush
- Disposable microtome blades
- Hot plate
- Hot water bath
- Pathn Situ positively charged slides
- Pressure cooker (5 Liters)
- Measuring Jars
- Coplin Jars
- Electronic Timer
- Absorbent wipes
- Coverslip for slides
- Binocular Light Microscope (Olympus CX21i)
- Micropipette
- Micropipette tips
- Rectangular steel trough
- Tweezer
- Induction stove
- Incubator (Hitech Equipments)

- Liquid repellent slide marking pen
- Deparaffinization stainless steel staining trough and rack
- pH meter (E1 digital pH meter)
- A DELTA PLAN2 AP40 Trinocular Light Microscope with camera Head

ANTIBODIES:

1. Primary antibody

(a) Anti p-mTOR (Ser 2448) Rabbit polyclonal Antibody – Bioss Antibodies U.S.A

2. Secondary kit (PolyExcel HRP/DAB Detection System) – Pathn Situ Biotechnologies Private Limited

(a) PolyExcel H_2O_2

(b) PolyExcel Target Binder

(c) PolyExcel Poly HRP

(d) PolyExcel stun DAB – Chromogen

(e) PolyExcel stun DAB – Buffer

REAGENTS USED:

- Tris Buffer – 50X concentration (Pathn Situ Biotechnologies Private Limited)
- Immuno wash Buffer – 25X concentration (Pathn Situ Biotechnologies Private Limited)
- Distilled water
- Xylene

- 100 % Ethanol
- 95 % Ethanol
- 70 % Ethanol
- 50 % Ethanol
- Harris Hematoxylin
- DPX Mountant

IMMUNOHISTOCHEMISTRY PROCEDURE:

- Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on charged slides and incubated for 45 minutes at 60°C
- For Deparaffinization & Rehydration:

1.Slides were washed two times in xylene for 3 minutes each time at room temperature(RT).

2. Slides were washed in xylene 1:1 with 100% ethanol for 3 minutes at RT.

3.Slides were washed two times in 100% ethanol for 3 minutes at RT.

4.Slides were washed two times in 95% ethanol for 3 minutes at RT.

5.Slides were washed in 70% ethanol for 3 minutes at RT.

6.Slides were washed in 50% ethanol for 3 minutes at RT.

7.Slides were rinsed gently with running distilled water for 5 minutes at RT.

- Antigen retrieval done for 15- 20 minutes (up to 3 whistles in pressure cooker)
- Cooled for minimum of 30 minutes
- Washed in distilled water, 2 changes, 2 minutes each

- Washed in PBS / TBS for 2 minutes
- Circles were marked enclosing the section using liquid repellent pen
- Endogenous peroxidase blocking was done by adding PolyExcel H_2O_2 on the section, keep for 30 minutes
- Washed in wash buffer for 5 minutes, 3 changes
- Primary antibody was added and kept for overnight incubation in a moist chamber
- Washed in wash buffer for 5 minutes, 3 changes
- Poly Excel Target Binder reagent was added and incubated for 30 minutes
- Washed in wash buffer for 5 minutes, 3 changes
- Poly excel HRP was added and incubated for 45 minutes
- DAB solution was prepared (1 ml of DAB buffer + 1 drop DAB chromogen, mix well)
- Washed in wash buffer for 5 minutes, 3 changes
- Working DAB chromogen was added and kept for 10 minutes, then washed in distilled water.
- Counterstained with hematoxylin for 30 seconds
- Washed in running tap water for 5 minutes
- Dehydrated through successive changes of alcohol and clear with xylene
- Dried and mounted with DPX

POSITIVE CONTROL

Positive control section includes human testis for p-mTOR and was treated in the same manner as the test groups.

NEGATIVE CONTROL

One section of test sample was selected and treated in the same manner as the test groups except that, the primary antibody was omitted.

Figure 2: Primary and Secondary Antibody kit

Primary antibody p-mTOR (Rabbit polyclonal Antibody) &

Antibody diluent

Secondary kit

[H2O2, Target Binder, Poly HRP]

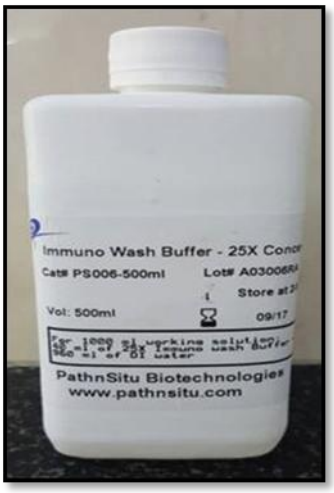
DAB Chromogen

and DAB buffer

Figure 3: Reagents used

Tris – EDTA

Hematoxylin



Wash Buffer

Figure 4: Equipments used

Deparaffinization stainless steel staining trough and rack

Micropipette

Incubator

Reagent blocker

Microtome

Microscope

pH meter

Moist chamber

Electronic Timer

Induction stove and Pressure cooker

RESULTS

The study group included formalin fixed paraffin embedded tissue blocks of normal buccal mucosa and Oral Squamous Cell Carcinoma (OSCC). It was divided in to 10 cases of normal buccal mucosa,21 cases of Oral Squamous Cell Carcinoma of which 7 cases of Well differentiated Squamous Cell Carcinoma,7 cases of Moderately differentiated Squamous Cell Carcinoma and 7 cases of Poorly differentiated Squamous Cell Carcinoma.

A total of 31 cases (n=31) were examined for the Immunohistochemical expression of p-mTOR which includes normal buccal mucosa (n=10) and OSCC (n=21).

Kruskall Wallis test was done for analysing the immunohistochemical expression and area of staining for p-mTOR.

Evaluation & Comparison of Immunohistochemical expression of p-mTOR in different grades of Oral Squamous Cell Carcinoma

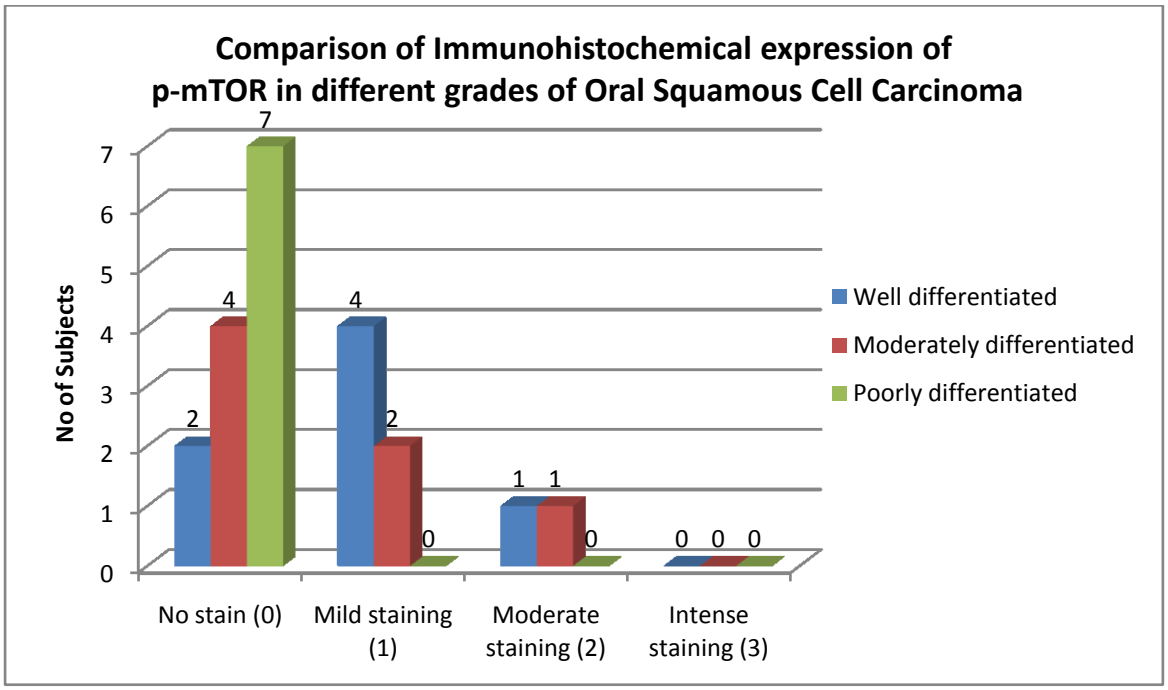
Out of 7 Cases of well differentiated squamous Cell Carcinoma, the immunoreactivity for intensity of staining p-mTOR showed positive expression in 5 cases (71%) and 2 cases (28%) did not show any expression. Among the positive 5 cases (71%), 4 cases (57%) showed mild staining,1 case (14%) showed moderate intensity of staining and none of the case (0%) showed intense staining. Out of 7 Cases of Moderately differentiated squamous Cell Carcinoma, the immunoreactivity for intensity of staining p-mTOR showed positive expression in 3 cases (42%) and 4 cases (57%) did not show any expression. Among the positive 3 cases (42%), 2 cases (28%) showed mild staining,1 case (14%) showed moderate intensity of staining and none of the case (0%) showed intense staining. Out of 7 Cases of Poorly differentiated Squamous Cell Carcinoma none of the cases (0%) showed positivity (Table 1& Graph 1).

Table 1: Evaluation & Comparison of immunohistochemical expression of p-mTOR in different grades of Oral Squamous Cell Carcinoma

GROUPS	NO OF CASES	SCORES				MEDIAN STAINING INTENSITY	IQR VALUE	p-VALUE
		No stain (0)	Mild staining (1)	Moderate staining (2)	Intense staining (3)			
Well differentiated	7	2 (28%)	4 (57%)	1(14%)	0 (0%)	1	1	**0.03**
Moderately differentiated	7	4 (57%)	2 (28%)	1 (14%)	0 (0%)	0	1	
Poorly differentiated	7	7 (100%)	0 (0%)	0 (0%)	0 (0%)	0	0	

Comparison of intensity of staining is **statistically significant** across these groups as p-value is **0.03** (with p-value <0.05)

Graph 1: Evaluation & Comparison of immunohistochemical expression of p-mTOR in different grades of Oral Squamous Cell Carcinoma



Evaluation & Comparison of immunohistochemical expression of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa

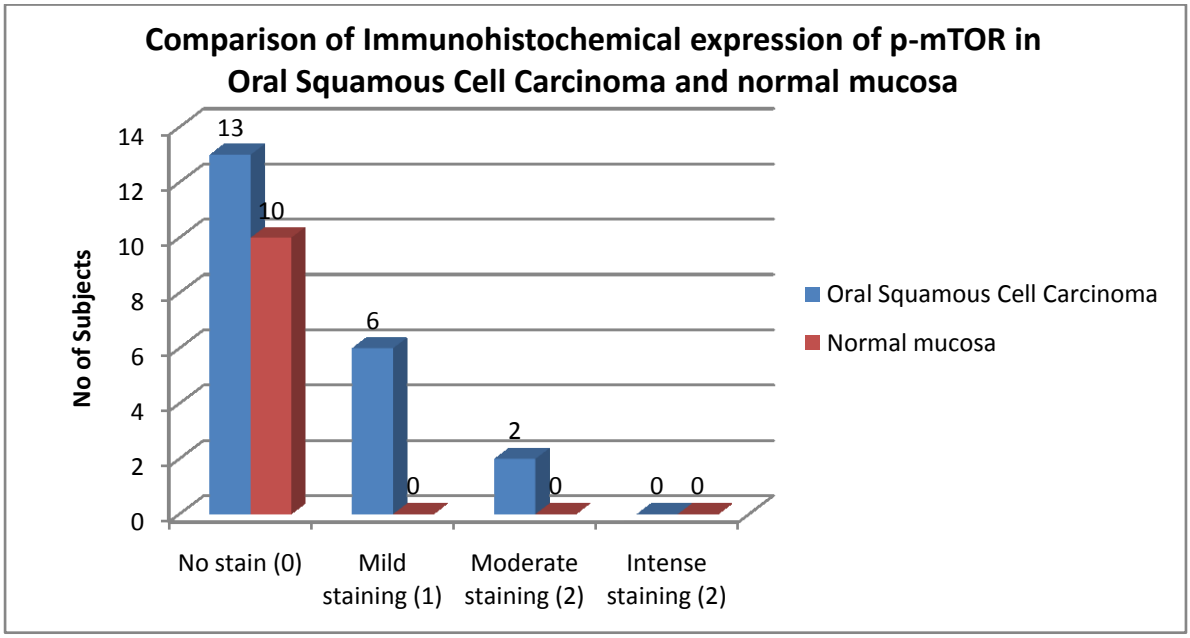
Out of 21 Cases of Oral Squamous Cell Carcinoma, the immunoreactivity for intensity of staining p-mTOR showed positive expression in 8 cases (38%) and 13 cases (61%) did not show any expression. Among the positive 8 cases (38%), 6 cases (28%) showed mild staining,2 cases (9%) showed moderate intensity of staining and none of the case (0%) showed intense staining. Out of 10 Cases of normal mucosa, none of the cases (0%) showed positivity (Table 2 & Graph 2).

Table 2: Evaluation & Comparison of Immunohistochemical expression of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa

GROUPS	NO OF CASES	SCORES				MEDIAN STAINING INTENSITY	IQR VALUE	p-VALUE
		No stain (0)	Mild staining (1)	Moderate staining (2)	Intense staining (3)			
Oral Squamous Cell Carcinoma	21	13 (61%)	6(28%)	2 (9%)	0(0%)	4	2	**0.003**
Normal mucosa	10	10 (100%)	0(0%)	0(0%)	0((0%)	0	0	

Comparison of intensity of staining is **statistically significant** across these groups as p-value is **0.003** (with p-value <0.05)

Graph 2: Evaluation & Comparison of Immunohistochemical expression of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa



Evaluation & Comparison of area of staining of p-mTOR in different grades of Oral Squamous Cell Carcinoma:

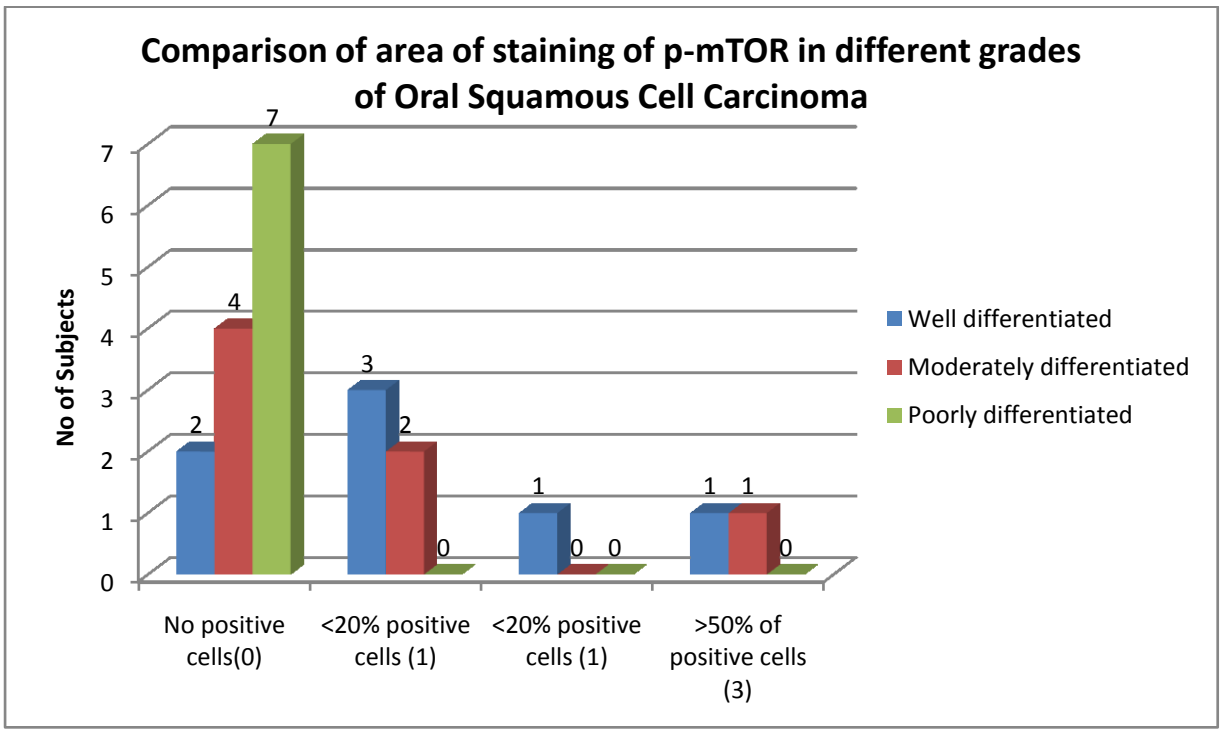
Out of 7 Cases of Well differentiated Squamous Cell Carcinoma, the immunoreactivity for area of staining of p-mTOR showed positive expression in 5 cases (71%) & 2 cases (28%) did not show any expression. Among the positive 5 cases (71%), 3 cases (42%) showed <20% of positive cells,1 case (14%) showed 20-50% of positive cells and 1 case (14%) showed >50% of positive cells. Out of 7 Cases of Moderately differentiated squamous Cell Carcinoma, the immunoreactivity for area of staining of p-mTOR showed positive expression in 3 cases (42%) and 4 cases (57%) did not show any expression. Among the positive 3 cases (42%), 2 cases (28%) showed <20% of positive cells and 1 case (14%) showed > 50% of positive cells. Out of 7 Cases of Poorly differentiated Squamous Cell Carcinoma none of the cases (0%) showed positivity.

Table 3: Evaluation & Comparison of area of staining of p-mTOR in different grades of Oral Squamous Cell Carcinoma:

GROUPS	NO OF CASES	SCORES				MEDIAN AREA OF STAINING	IQR VALUE	p-VALUE
		No positive cells (0)	<20% of positive cells (1)	20-50% of positive cells (2)	>50% of positive cells (3)			
Well differentiated	7	2(28%)	3(42%)	1(14%)	1(14%)	1	2	**0.03**
Moderately differentiated	7	4(57%)	2(28%)	0(0%)	1(14%)	0	1	
Poorly differentiated	7	7(100%)	0(%)	0(0%)	0(0%)	0	0	

Comparison of area of staining is **statistically significant** across these groups as p-value is **0.03** (with p-value <0.05)

Graph 3: Evaluation & Comparison of area of staining of p-mTOR in different grades of Oral Squamous Cell Carcinoma



Comparison of area of staining of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa:

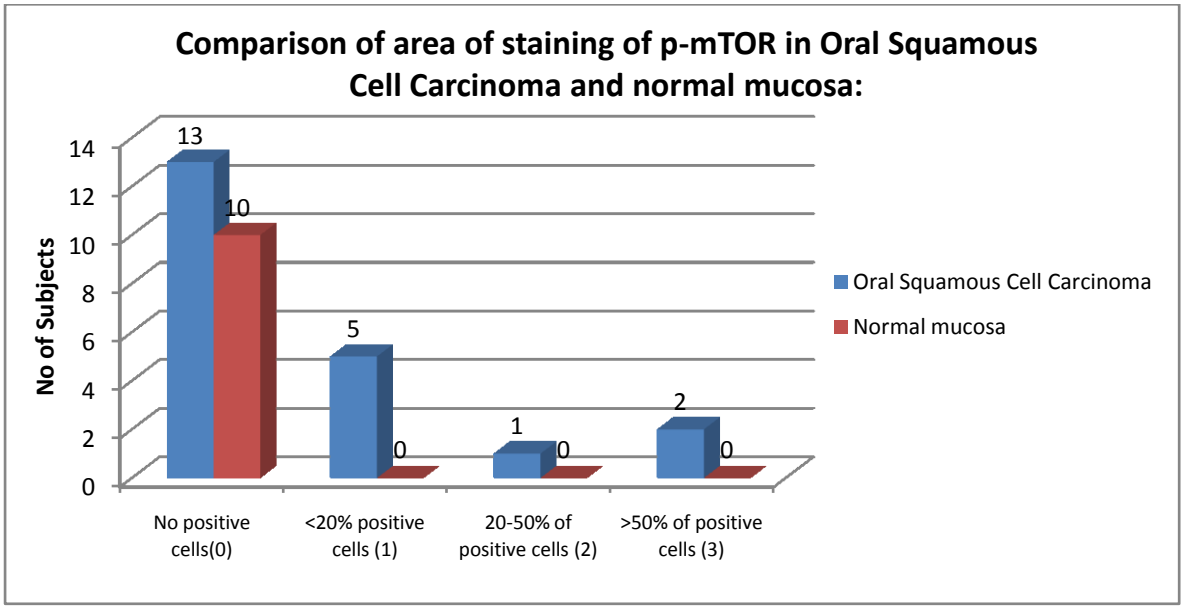
Out of 21 Cases of Oral Squamous Cell Carcinoma, the immunoreactivity for area of staining of p-mTOR showed positive expression in 8 cases (38%) & 13 cases (61%) did not show any expression. Among the positive 8 cases (38%), 5 cases (23 %) showed <20% of positive cells,1 case (14%) showed 20-50% of positive cells and 2 cases (9%) showed >50% of positive cells. Out of 10 normal mucosa none of the cases (0%) showed positivity.

Table 4: Comparison of area of staining of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa:

GROUPS	NO OF CASES	SCORES				MEDIAN AREA OF STAINING	IQR VALUE	p-VALUE
		No positive cells (0)	<20% of positive cells (1)	20-50% of positive cells (2)	>50% of positive cells (3)			
Oral Squamous Cell Carcinoma	21	13(61%)	5(23%)	1(14%)	2(28%)	3	3	**0.003**
Normal mucosa	10	10(100%)	0(0%)	0(0%)	0(0(%)	0	0	

Comparison of area of staining is **statistically significant** across these groups as p-value is **0.003** (with p-value <0.05)

Graph 4: Comparison of area of staining of p-mTOR in Oral Squamous Cell Carcinoma and normal mucosa:



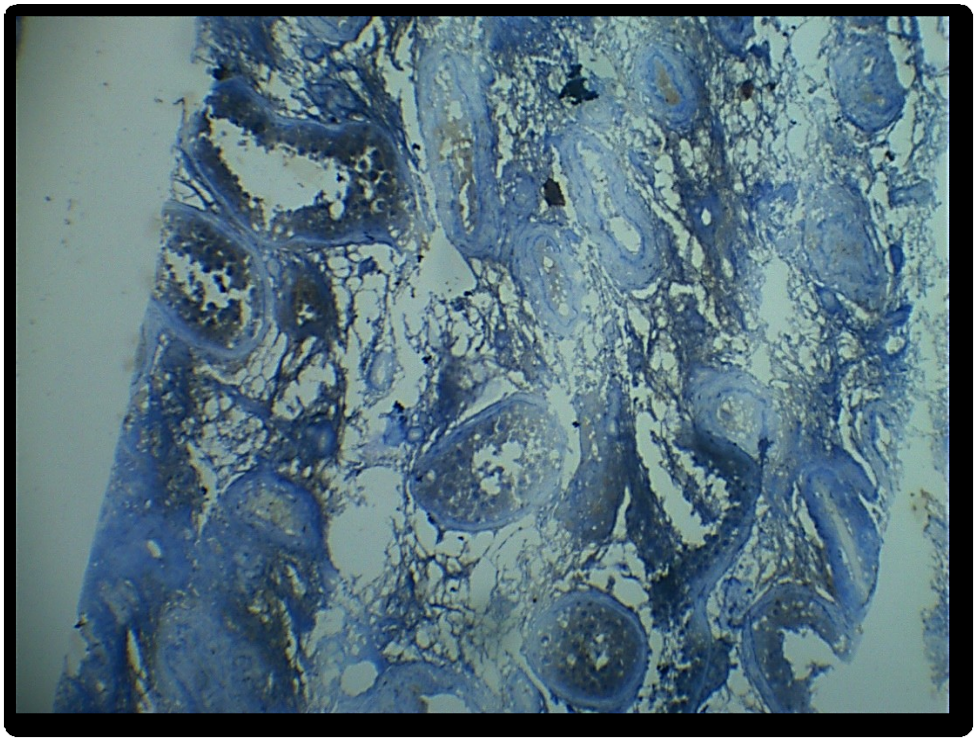


Figure 5: Low power view (10x) showing showing expression of p-mTOR in positive control (Testis)

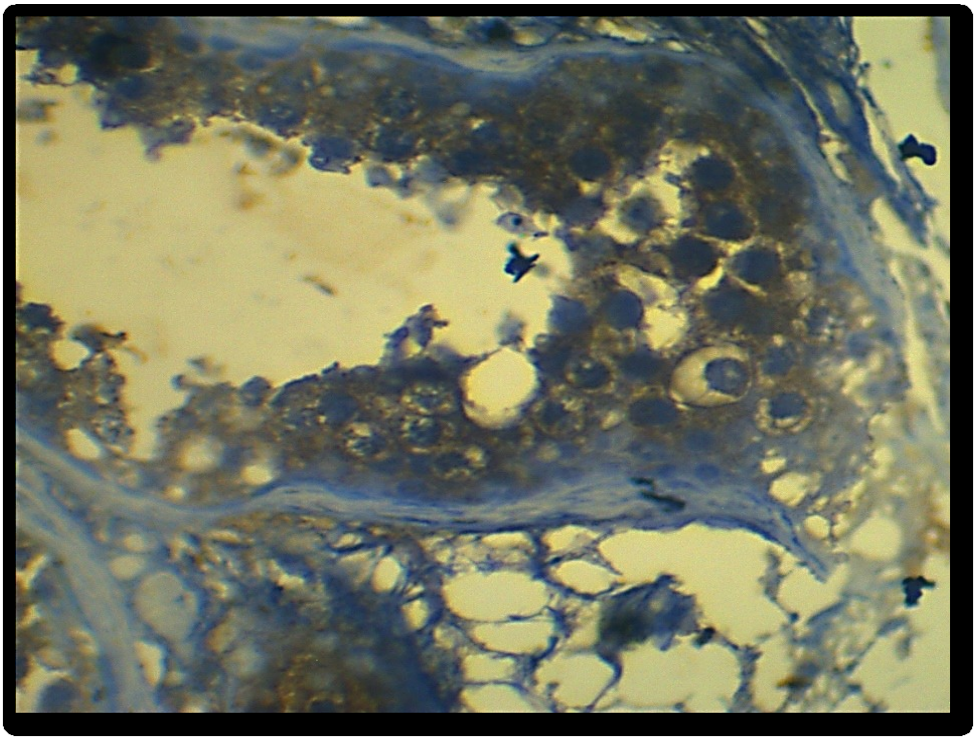


Figure 6: High power view (40x) showing expression of p-mTOR in positive control (Testis)

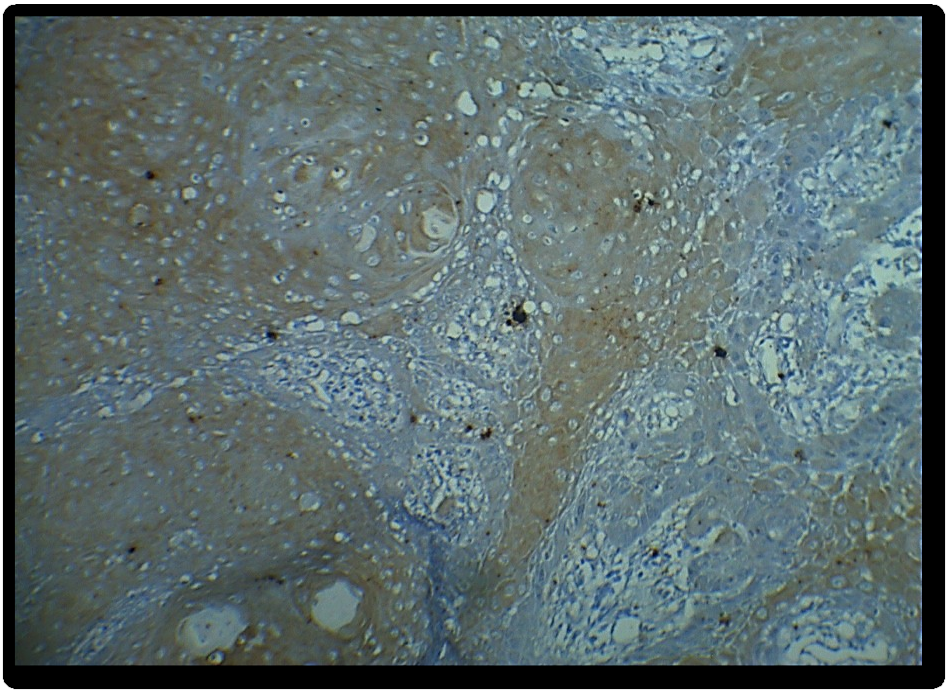


Figure 7: Low power view (10x) showing moderate expression of p-mTOR in Well differentiated Squamous Cell Carcinoma.

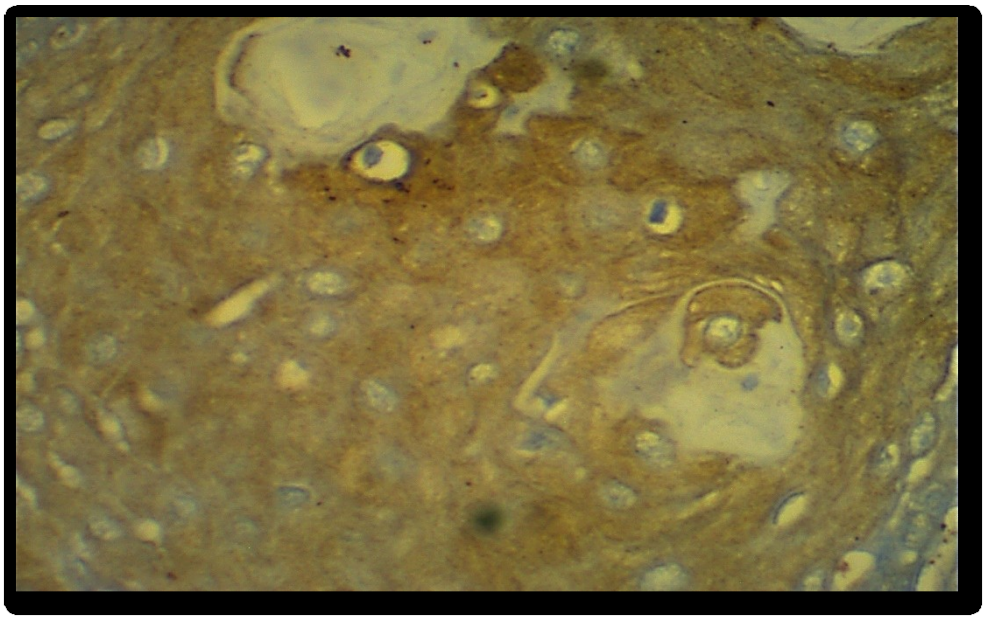


Figure 8: High power view (40x) showing moderate expression of p-mTOR in Well differentiated Squamous Cell Carcinoma.

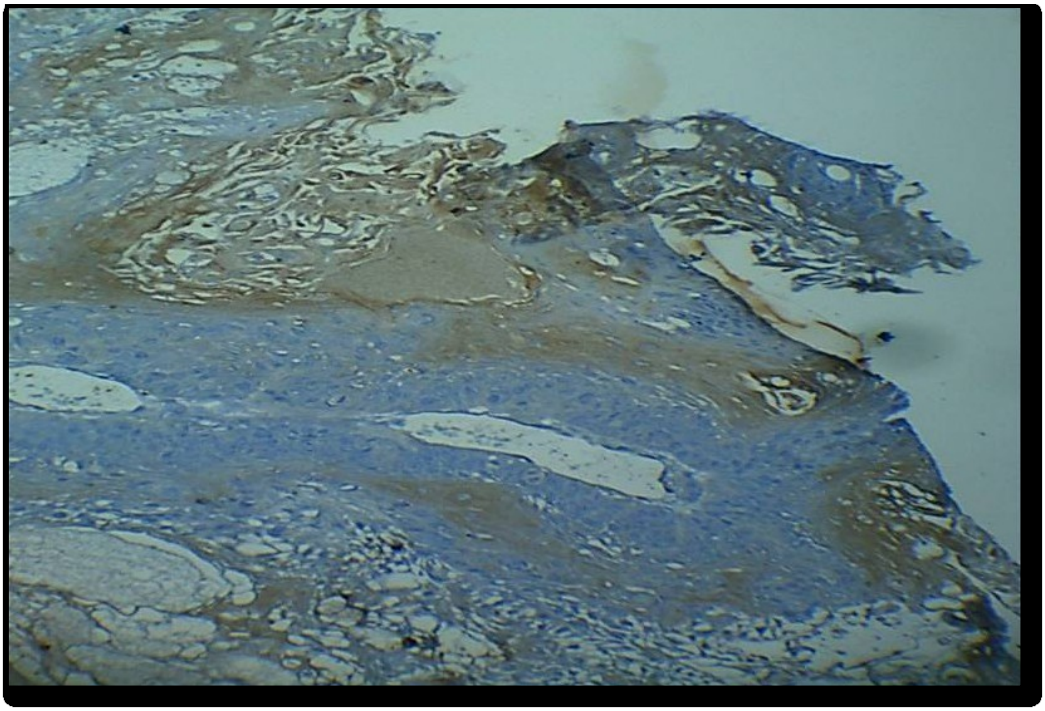


Figure 9: Low power view (10x) showing moderate staining in Moderately differentiated Squamous Cell Carcinoma

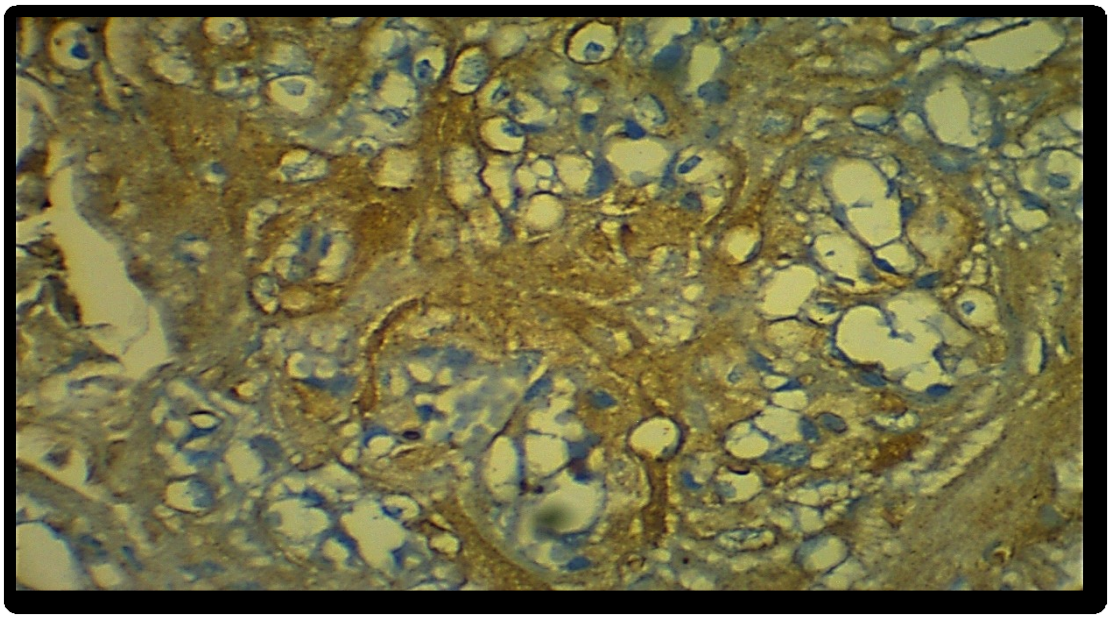


Figure 10: High power view (40x) showing moderate staining in Moderately differentiated Squamous Cell Carcinoma

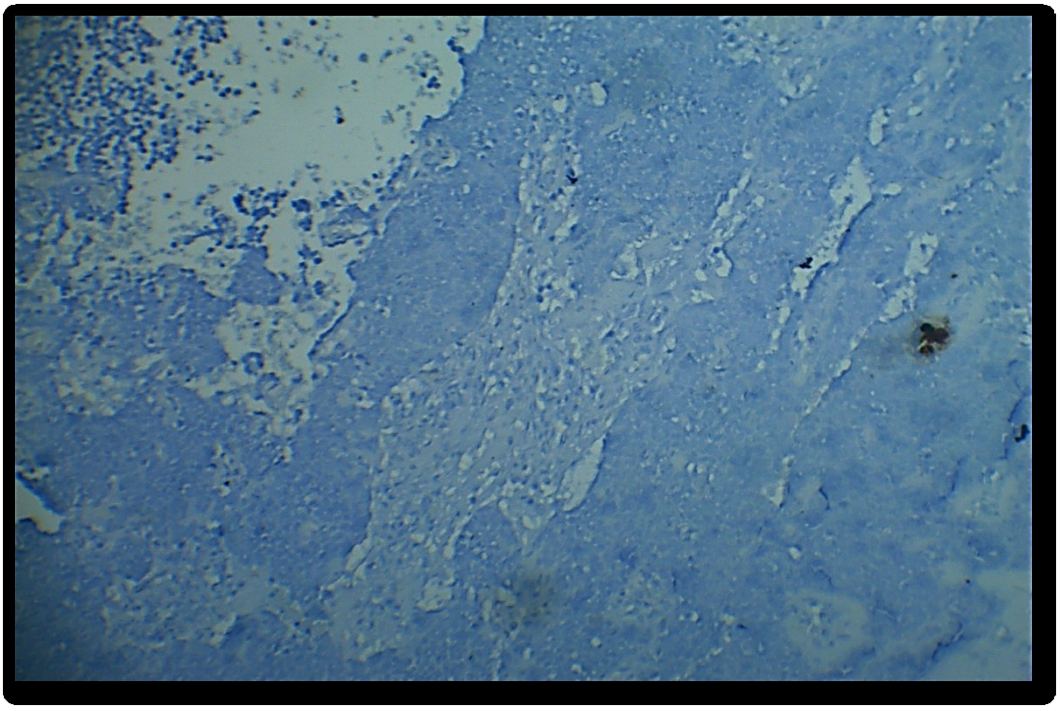


Figure 11: Low power view (10x) showing negative staining in Poorly differentiated Squamous Cell Carcinoma

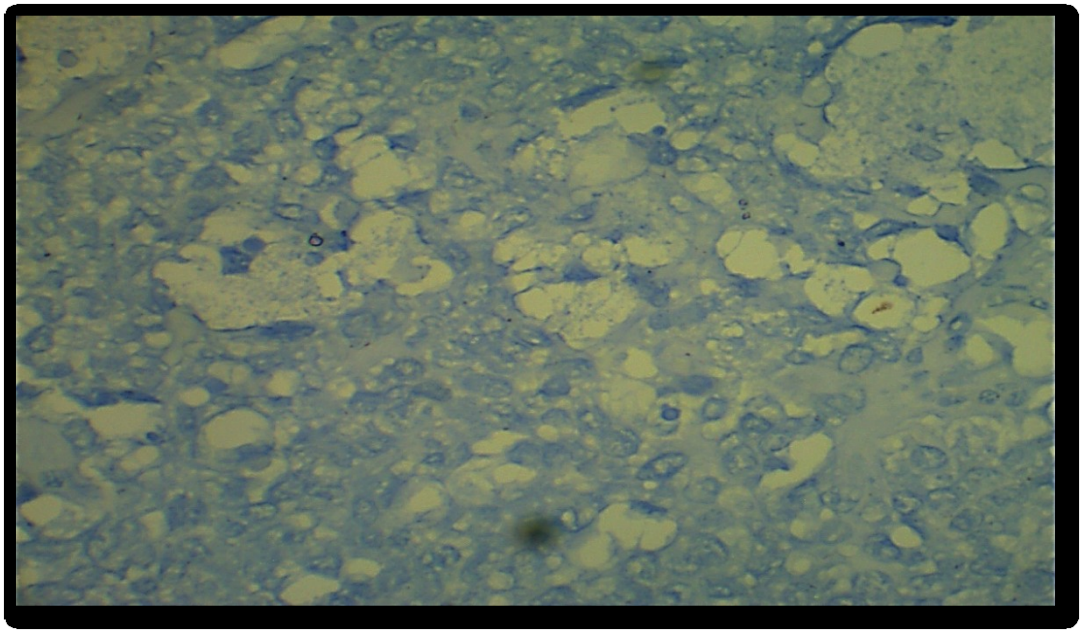


Figure12: High power view (40x) showing negative staining in Poorly differentiated Squamous Cell Carcinoma

DISCUSSION

Oral Squamous Cell Carcinoma constitutes about 95% of all forms of head & neck cancer[3]. Its incidence is high in many countries [8] and its incidence is increased by 50% for the last decade[3]. The highest incidence and prevalence of OSCC is established in the Indian subcontinent where the risk of OSCC occurrence is increased by the prevalent habits of chewing tobacco, betel quid, areca nut and the regular drinking of alcoholic beverages[18].Oral tumorigenesis is a multistep process in which cells accumulate disparate complement mutations which allows them to escape from normal cellular and environmental constraints on proliferation[3].It is caused due to sequential accumulation of multiple gene alterations. The aberrations may occur in a number of cellular signaling pathways. One such key pathway is PI3/Akt/mTOR pathway. Molecular aberrations in this pathway may act as prognostic markers or targets for molecular therapies[7].

m-TOR is a serine threonine kinase and it is a member of PI-3 kinase family[14,43]. It plays an important role in maintaining the cell growth, cellular nutrient and energy status[44]. The significance of phosphorylated mTOR remains unceratin[7]. So, in the current study, Immunohistochemical expression of p-mTOR in different grades of OSCC was evaluated in order to assess the potential contribution of Akt /mTOR/ pS6 signaling pathway aberrations in OSCC.

In the present study, a total of 31 samples, out of which 21 cases of Oral Squamous Cell Carcinoma and 10 cases of normal buccal mucosa were evaluated for the expression of p-mTOR using standard IHC procedure with anti p-mTOR antibody (rabbit polyclonal antibody-Bioss USA). In this study, OSCC was graded according to Broder's classification (1920) as Grade I – Well differentiated (75 to 100% of cells are differentiated), Grade II – Moderately differentiated (50 to 75 % of cells are differentiated) and Grade III – Poorly differentiated (25 to 50% are differentiated)[16].

In our study, on evaluating and comparing the p-mTOR expression in different grades of OSCC, the staining intensity was found to be statistically significant between the different grades of OSCC (p value = 0.03). This is in accordance with the study conducted by **Geogios Prodromidis *et al.***, **Hyun Lee *et al.***, **Noske Aurelia *et al.*, and Boone *et al.***

Out of 7 cases of Well differentiated OSCC, 5 cases (71%) showed positive staining, out of 7 cases of Moderately differentiated OSCC, 3cases (42%) showed positive staining. All 7 cases of Poorly differentiated OSCC showed negative expression (Table 1& Graph 1).

These results were similar to the study conducted by **Hyun Lee *et al.*,** (p=0.008) who assessed the expression of p-mTOR in Small Cell Lung Cancer[45].

Noske Aurelia *et al.*, (2008) investigated the activation of mTOR in a subgroup of ovarian Carcinomas and corelated it with prognosis. They concluded that for the first-time that higher p-mTOR expression was associated with primary Ovarian Carcinoma and was significantly associated with better overall survival (p=0.003). Even in our present study higher p-mTOR expression is associated with low grade tumours. The results of our study matched with this study[46].

In the present study, out of 7 cases of Poorly differentiated OSCCs, none of the case showed the expression for p-mTOR. These results were inconsistent with the study conducted by **Boone *et al.*,** who assessed the expression of p-mTOR in squamous cell carcinoma of Oesophagus. They concluded that activated mTOR expression is associated with lower degree of differentiation (p=0.024)[34].

mTOR is one of the major targets of activated Akt, which in turn regulates a number of downstream molecules such as ribosomal protein pS6, which controls the fundamental cell processes such as cell survival, proliferation, protein synthesis and angiogenesis[5,47]. Dysregulations in upstream and downstream molecules of mTOR signals may contribute to aberrations in Akt /mTOR /pS6 signaling pathway, which is highly conserved and controlled by multistep process[48,49,50]. Hence the result of our present study shows that the expression of mTOR plays a vital role in this signaling pathway of different grades of OSCC.

In our current study, on comparing the intensity of staining in between OSCC and normal buccal mucosa, the intensity of staining was found to be statistically significant across these groups (p value = 0.003) (Table 2 and Graph 2). These results were consistent with the study of **Wang Di *et al*., Clark Cheryl *et al*.**, **Sahin *et al*., & Xiao *et al*.**

Wang Di *et al.*, (2011) investigated the clinical significance of m-TOR and p-mTOR protein expression in human Colorectal Carcinomas. They obtained significant over expression of p-mTOR in Colorectal Adenocarcinomas than in normal mucosa samples. This is in accordance with our study, where significant expression of p-mTOR was found in OSCC when compared to normal mucosa (p=o0.007).They concluded that mTOR is highly associated with colorectal cancer and plays a key role in tumour carcinogenesis[37].

Clark Cheryl *et al*., (2010) evaluated the expression of p-mTOR and p-4EBP1 (translational factor) in Head & Neck Squamous Cell Carcinoma (HNSCC) samples and non-cancer mucosa samples. The results of their study indicated that higher expression seen in basal layer of cancer samples than in non-cancer oral mucosa samples (p=0.001). They concluded that m-TOR is dysregulated in HNSCC samples. The results of the current study were consistent with this study[33].

Sahin *et al.*, (2004) evaluated mTOR in primary Liver neoplasms and correlated their expression with clinicopathological parameters. Increased p-mTOR expression was seen in 15% of hepatocellular carcinoma (p=0.018). He concluded that the mTOR signaling pathway is known to be upregulated in various carcinoma including human Ovarian and Breast Carcinomas. Protein translation is highly regulated in cells, in part by the mTOR pathway, which provides nutrient and energy availability and phosphorylates p70S6 kinase (S6k) to promote protein translation. S6k in turn phosphorylates the 40s ribosomal subunit of protein S6 which leads to increased translation of mRNAs containing 5'-terminal oligopyrimidine tracts which code for ribosomal proteins and elongation factors which is necessary for protein translation. Thus, the mTOR pathway is an attractive target for cancer therapeutics as effective target for cancer therapeutics, as effective mTOR inhibitors such as Rapamycin block mTOR phosphorylation of S6k[51].

Xiao *et al.*, (2009) analysed the role of mTOR and p70S6k in pathogenesis and progression of gastric carcinomas. They found that expression of p-mTOR is high in gastric carcinoma ($p<0.05$). He concluded that mTOR plays a key role in cellular growth and homeostasis and its regulation is freely altered in tumours[52].

In our study, on evaluating and comparing the area of staining of p-mTOR in different grades of OSCC, the area of staining was found to be statistically significant between the different grades of OSCC (p value =0.03) (Table 3 & Graph 3). These results were consistent with the study conducted by **Stelloo *et al***., and **Afonso Julieta *et al.***

On comparing the area of staining, out of 7 cases of Well differentiated OSCC,5 cases (71%) showed positive stained cells. Out of 7 cases of Moderately differentiated OSCCs, 3 cases (42%) showed positive stained cells. Out of 7 cases of Poorly differentiated SCCs none of the cases showed positive stained cells.

Stelloo *et al.*, (2015) assessed the mTOR pathway activation in human Prostate Adenocarcinoma. They correlated the expression of mTOR with clinical parameters. Low mTOR expression (based on positive % of tumour cells) is associated with high pathologic stage (p=0.01). The results of our study are consistent with the study of **Stelloo *et al*** [53]**.**

Afonso Julieta *et al*., (2014) investigated the p- mTOR in non - tumour and tumour bladder urothelium. The results of their study are, the expression of p-mTOR decreased with increasing stage (based on both intensity of staining and % of positive tumour cells) and the mTOR expression was lost with enhanced tumour aggressiveness (p=0.087). The results of the present study are consistent with the

study of **Afonso Julieta *et al*** as we obtained negative stain for Poorly differentiated SCCs[54].

In our study, on comparing the area of staining in between OSCC and normal buccal mucosa, the area of staining was found to be statistically significant across these groups (p value =0.003) (Table 4 and Graph 4). These results were consistent with the study of **Altomare *et al*.,** and **Min Li *et al.***

Altomare *et al.*, (2004) evaluated Akt and mTOR phosphorylation in Ovarian cancers. In their study 87% of Ovarian Cancers are associated with active mTOR expression. He concluded that activation of PI3/Akt pathway contribute to tumorigenesis (p=0.03). The results of our study are consistent with this study[55].

Min Li *et al.*, (2012) evaluated the Immunohistochemical expression of mTOR and PTEN in gastric carcinoma and in normal mucosa. They found that the mTOR was distributed mainly in the cytoplasm of Gastric Carcinoma tissues (based on both intensity of staining and % of positive tumour cells) and the normal mucosa showed negative results (p<0.01). The results of our present study are consistent with this study [27].

We analysed the expression of p-mTOR as the first attempt in between different grades of OSCC and compared the expression with normal mucosa. Our study is a small initiative to find out the significance of p-mTOR expression in different grades of OSCC.

Earlier studies have shown that the expression of p-mTOR only in Breast Carcinoma, Small Cell Lung Cancer, Oesophageal Carcinomas, Cervical carcinomas, Gastric Carcinomas & Colorectal Carcinomas. But our present study reveals the significant expression of p-mTOR in Akt /mTOR/pS6 signaling pathway which act as a prognostic marker or target for molecular therapies.

SUMMARY AND CONCLUSION

The aim of this study was to analyse the Immunohistochemical expression of p-mTOR in biopsy samples of different grades of OSCC in comparison with normal mucosa.A total of 31 samples, out of which 21 cases of OSCC and 10 cases of normal buccal mucosa were taken from archival blocks. Immunohistochemical expression of p-mTOR were studied by analysis of intensity of staining and area of staining.

From the present study done with anti-p-mTOR, following conclusions were drawn:

- On comparing the intensity of staining between different grades of OSCC the value is statistically significant (p=0.03)
- Statistically significant difference (p=0.003) exists for intensity of staining in between OSCC and normal mucosa.
- On comparing the area of staining between different grades of OSCC the value is statistically significant (p=0.03).
- Statistically significant difference (p=0.003) exists for area of staining in between OSCC and normal mucosa.

Our present study reveals the significant expression of p-mTOR in Akt /mTOR/pS6 signaling pathway which act as a prognostic marker or target for molecular therapies. Further research with a larger number of sample size with clinicopathologic correlation and long term follow up will emphasize more towards the use of mTOR as a prognostic

marker. It will also be more interesting to correlate the presence and frequency of other molecular aberrations such as p-Akt and pS6 to trace out the role of entire Akt /mTOR/pS6 signaling pathway in OSCC which will also be useful for the development of new therapeutic strategies targeting on mTOR pathway.

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[Primary Antibody]

mTOR (Ser2448) Polyclonal Antibody

Size: 20ul
Conc: 1ug/ul

Host:	Rabbit
Target Protein:	mTOR Ser2448
Specificity:	This phosphorylation site is homologous across the listed species.
Modification Site:	Ser2448
Clonality:	Polyclonal
Isotype:	IgG
Immunogen Range:	2420-2470/2549
Entrez Gene:	2475
Swiss Prot:	P42345
Source:	KLH conjugated synthetic phosphopeptide derived from human mTOR around the phosphorylation site of Ser2448
Purification:	Purified by Protein A.

Applications:
IHC-P(1:100-500)
IF(IHC-P)(1:50-200)

Cross Reactive Species:
Human
Mouse
Rat

Storage: Aqueous buffered solution containing 1% BSA, 50% glycerol and 0.09% sodium azide. Store at -20°C for 12 months.

Background: Serine/threonine protein kinase which is a central regulator of cellular metabolism, growth and survival in response to hormones, growth factors, nutrients, energy and stress signals. MTOR directly or indirectly regulates the phosphorylation of at least 800 proteins. Functions as part of 2 structurally and functionally distinct signaling complexes mTORC1 and mTORC2 (mTOR complex 1 and 2). Activated mTORC1 up-regulates protein synthesis by phosphorylating key regulators of mRNA translation and ribosome synthesis. This includes phosphorylation of EIF4EBP1 and release of its inhibition toward the elongation initiation factor 4E (eiF4E). Moreover, phosphorylates and activates RPS6KB1 and RPS6KB2 that promote protein synthesis by modulating the activity of their downstream targets including ribosomal protein S6, eukaryotic translation initiation factor EIF4B, and the inhibitor of translation initiation PDCD4. Stimulates the pyrimidine biosynthesis pathway, both by acute regulation through RPS6KB1-mediated phosphorylation of the biosynthetic enzyme CAD, and delayed regulation, through transcriptional enhancement of the pentose phosphate pathway which produces 5-phosphoribosyl-1-pyrophosphate (PRPP), an allosteric activator of CAD at a later step in synthesis, this function is dependent on the mTORC1 complex. Regulates ribosome synthesis by activating RNA polymerase III-dependent transcription through phosphorylation and inhibition of MAF1 an RNA polymerase III-repressor. In parallel to protein synthesis, also regulates lipid synthesis through SREBF1/SREBP1 and LPIN1. To maintain energy homeostasis mTORC1 may also regulate mitochondrial biogenesis through regulation of PPARGC1A. mTORC1 also negatively regulates autophagy through phosphorylation of ULK1. Under nutrient sufficiency, phosphorylates ULK1 at 'Ser-758', disrupting the interaction with AMPK and preventing activation of ULK1.

PI 0041, Rev. D DCN: 2665 Effective Date: 05/24/2017

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BIOSCIENCE FOR THE WORLD

IVD

For In Vitro Diagnostic Use

ImmunoDetector Protein Blocker/Antibody Diluent

Intended Use *For In Vitro Diagnostic Use*

Summary And Explanation ImmunoDetector Protein Blocker/Antibody Diluent is used to dilute ascites, supernatants, purified antibodies, and polyclonal antibodies. The reagent is designed to minimize the non-specific reaction that may be caused by non-specific antibody interactions and encourages specific antigen-antibody binding.

Presentation ImmunoDetector Protein Blocker/Antibody Diluent contains phosphate TBST, pH 7.6, with bovine serum albumin, and preserved with sodium azide as an anti-microbial. It is provided in liquid form ready-to-use.

Availability

Catalog No.	Concentration	Volume
BSB 0113	Ready-to-use	15 mL
BSB 0040	Ready-to-use	50 mL
BSB 0041	Ready-to-use	100 mL
BSB 0114	Ready-to-use	200 mL
BSB 0115	Ready-to-use	1000 mL

Storage Store at 2-8°C **Stability** 3 years

The ImmunoDetector Protein Blocker/Antibody Diluent is stable at room temperature for up to **3** years from when originally produced (see expiration date on product label). This product is stable up to the expiration date on the label. Do not use this product after the expiration date. Adhere to all local laws when disposing of this product.

Preparation of Working Solutions

The ImmunoDetector Protein Blocker/Antibody Diluent is a ready-to-use working solution and requires no further preparation.

Recommended Protocol

When diluting antibodies, add antibody to the diluent, not diluent to the antibody. Addition of the antibody to the mixing vessel before the diluent can cause contamination of the diluent if multiple dispenses are necessary.

PathnSitu

Rev: A
Release Date: 03/13/2014
IVD

PolyExcel HRP/DAB Detection System-TWO STEP
Universal kit for Mouse and Rabbit Primary Antibodies

Intended Use: For *In vitro* diagnostic use

PolyExcel detection system is intended to use with primary antibodies raised against **mouse** and **rabbit** for the qualitative identification of antigens by light microscopy in normal and pathological paraffin-embedded tissues, cryostat tissues or cell preparations.

Summary and Explanation: PathnSitu's highly sensitive and specific PolyExcel two step detection system is non-biotin, micro-polymer based detection system which significantly reduce or shows no back ground on tissues containing high levels of avidin, biotin ex: Kidney, Liver and lymphoid tissues. This system is based on an HRP labeled polymer, which is conjugated with secondary antibodies.

Principal of procedure: Incubating the specimen for 5–10 minutes with H_2O_2 quenches any endogenous peroxidase activity. The specimen is then incubated with respective diluted mouse or rabbit primary antibody, followed by incubation with the PolyExcel Target Binder for 10 minutes then followed by a PolyExcel HRP labeled polymer using recommended 10 minutes incubation. Staining is completed by 5–10 minutes incubation with 3,3'-diaminobenzidine (DAB) substrate-chromogen which results in a brown-colored precipitate at the antigen site (DAB is a potential carcinogen; Please take appropriate precautions).

Kit Contents:
PathnSitu PolyExcel detection kit supplied as 3 pack sizes. Details below:

Description	Cat# / Pack Size	Kit Contents
PolyExcel HRP/DAB Detection System	PEH002-6ml	PolyExcel H2O2 PolyExcel Target Binder PolyExcel PolyHRP PolyExcel Stunn DAB Substrate Buffer PolyExcel Stunn DAB Substrate Chromogen
	PEH002-50ml	
	PEH002-100ml	

Materials required but not supplied:
1. Positive charged slides (PathnSitu Cat# PS011-72)
2. Control Tissues
3. Xylene
4. Isopropyl alcohol
5. DI Water
6. Hematoxylin
7. Cover glass
8. Mounting media
9. Antigen retrieval buffers (PathnSitu Cat# PS007, PS008, PS009)
10. Immuno wash Buffer (PathnSitu Cat# PS006)

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Prof.Dr.T.Ramakrishnan, MDS

This ethical committee has undergone the research protocol submitted by **ABIRAMI.M** Post Graduate Student, Dept of **ORAL PATHOLOGY** under the title "**IMMUNOHISTOCHEMICAL EVALUATION OF p-mTOR (PHOSPHORYLATED MAMMALIAN TARGET OF RAPAMYCIN) IN ORAL SQUAMOUS CELL CARCINOMA",** Reference No**: 2015-MD-Br VI-DEV-05/APDCH** under the guidance of **DR.M.DEVI, MDS.,** for consideration of approval to proceed with the study.

This committee has discussed about the material being involved with the study, the qualification of the investigator, the present norms and recommendation from the Clinical Research scientific body and comes to a conclusion that this research protocol fulfils the specific requirements and the committee authorizes the proposal.

Date:

CHAIR PERSON

- Inform IEC/IRB immediately in case of any issue(s) / adverse events.
- Inform IEC/IRB in case of any change of study procedure, site and investigator.
- Annual report to be submitted to IEC/IRB.
- Members of IEC/IRB have right to monitor the trial with prior intimation.